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**PLASMA METABOLOMIC PROFILING BASED DETECTION OF DRUG SPECIFIC RESPONSES
TO DIFFERENT BOVINE GROWTH PROMOTING REGIMES**

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ABBREVIATIONS

EDTA – Ethylenediaminetetraacetic acid; MW – molecular weight; m/z – mass to charge
ratio; AMRTP – accurate mass and retention time pair; QC – quality control; FC – Fold
Change; RSD – relative standard deviation; ANOVA – analysis of variance; UHPLC Ultra
High Performance Liquid Chromatography; MS: Mass Spectrometry; PCA – Principal
Component Analysis; OPLS-DA – Orthogonal Partial Least Squares Discriminant Analysis;
VIP – variable importance of projection.

ABSTRACT

The use of anabolic substances for growth promoting purposes in food producing animals is prohibited within the EU, yet ongoing applications of hormones such as oestradiol prove both difficult to detect and to distinguish from endogenous presence. Additionally, the misuse of glucocorticoid compounds (dexamethasone and prednisolone), which are permitted for therapeutic applications but can also promote improved animal health through long-term dosing, is reported to be increasing posing potential health concerns for consumers. Twenty-four male beef cattle were randomly assigned to four groups ($n=6$) for experimental treatment over 40 days consisting of a control untreated group, and three treatment groups administered oestradiol, dexamethasone or prednisolone at levels known to reflect growth promoting practices. Untargeted metabolomic profiling of plasma collected from each animal midway through the study treatment period, were analysed by reverse phase separation employing an UHPLC-QToF-MS system operating in positive electrospray ionization mode. Metabolomics analysis revealed plasma metabolite perturbations common to all treated animals, with additional metabolites found to be specifically associated with the various differing growth promoting regimes. OPLS-DA modelling was used to discriminate plasma profiles of oestradiol, dexamethasone, or prednisolone from control untreated cohorts and found 56, 48 and 58 ions altered by the administered treatments respectively. This culminated in 99 shared ions which could distinguish between plasma samples from treated versus untreated animals. Additional assessment of the metabolites found ions which were significantly altered in comparison to control animals, of which 3, 11 and 8 ions were pertinent to oestradiol, dexamethasone or prednisolone administrations respectively. Incorporation of such markers to specific treatment types could be used at screening to facilitate further

confirmatory analysis. Putative identification of these ions demonstrated mainly lipid components responsible for the growth promoter metabolomic effects alongside novel biomarker responses.

KEYWORDS: Cattle/ Anabolic/ Blood/ Metabolite/ Screening/ Corticosteroid

HIGHLIGHTS

- Metabolomic profiling of bovine plasma outlines effect-based responses to growth promoter abuse and increases the capability to detect unsafe meat.
- Predictive modelling based on the plasma metabolome can differentiate between samples acquired from treated and untreated animals profiles based on common metabolites of interest.
- Monitoring of plasma metabolite markers specific to oestradiol, dexamethasone or prednisolone can be employed to identify the type of growth promoting regime employed
- Biomarkers responses reveal the distinct modes of action of the growth promoting agents relevant to their metabolic effects.

1. INTRODUCTION

The implementation of screening based testing for detection of drug residues in food producing animals is a required action within the European Union as stipulated in EC Regulation 178/2002 (2002a). Testing is assigned through National Residue Control Plans (NRCP) coordinated by European Residue Laboratories (EURLs) and results are reported to the European Food Safety Authority (EFSA) for annual review. EURLs are required to test 0.4% of slaughtered cattle numbers to meet minimum legislative requirements (Directive, 1996), and while routine regulatory monitoring finds sufficient compliance (DAFM, 2015), additional random on-farm sampling indicates continued illicit use of chemical agents within beef producing animals (Leporati et al. 2015; Imbimbo et al. 2012; Chiesa et al. 2017). The financial gains arising from illegal growth promoting administration encourages their use and exposes consumers to toxicological risk from contaminated food materials due to a combination of irregular drug use and ineffective testing (Ronquillo et al. 2017).

Current test methods are dependent on direct detection analysis of known compounds with confirmatory analysis typically reliant on gas (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) methods. Despite improved sensitivity through progress in these advanced technologies, analytical challenges to the detection of growth promoter use persist (Stolker et al. 2005; van Ginkel et al. 2016). These challenges include the detection of emerging unknown compounds, identification of drug use at low doses, and effective discrimination between endogenous forms of hormones and exogenous administrations either as therapeutics or for illicit purposes (Mooney et al. 2009; Pinel et al. 2010; Courtheyn et al. 2002). The latter includes glucocorticoid and oestradiol derivatives which are increasingly abused (EFSA, 2012; Sterk

et al. 2014) due to their natural presence which is indistinguishable from external application. In this way confirmatory methods have incorporated isotope ratio (IRMS) techniques to discriminate exogenous metabolites based on the ratio of $^{13}\text{C}/^{12}\text{C}$ (Janssens et al. 2013). However, such analyses are only available through confirmatory test methods and robust screening methods are needed. As such more research in this field is be directed towards assessment of an animal's biological response to drug administration as a feasible alternative approach to discriminate biomarkers significant to xenobiotic exposure (Nebbia et al. 2011). In this way, Marin et al. (2008) were able to discern dexamethasone administration in finishing bulls by monitoring blood parameters, whilst Mooney et al. (2009) and Doué et al. (2015) have demonstrated biochemical screening of sex-hormone and bone markers as indicative of steroid misuse.

The range of sample matrices available for anabolic screening tests varies and is dependent on the regulatory body requirements on whether the drug to be tested is acquired from live or slaughtered animals (Directive, 1996). External biological material such as urine, hair and blood can be sampled on farm, whilst consumable parts are only available after slaughter. Some metabolomic studies have been conducted with urine to distinguish treatment of oestradiol, β -agonist and prohormones (Dervilly-Pinel et al. 2011; Jacob et al. 2015; Courant et al. 2009; Rijk et al. 2009), however there are concerns of false positive results due to faecal contamination (Arioli et al. 2010) and also endogenous prednisolone levels caused by stress (Pompa et al. 2011). Similarly, hair analysis may be subject to environmental contamination and obscured by the method of drug delivery, whilst drug residues are known to diffuse rapidly (Vanhaeke et al. 2011). For the purpose of screening, blood can be collected on farm and Noppe et al. (2008) previously reported a higher occurrence of steroid hormones within the blood due to the circulating action

from anabolic tissues and metabolomic profiling has already revealed potential biomarkers within the plasma collected (Graham et al. 2012).

Metabolomic fingerprinting has been promoted as a non-targeted approach whereby the entire metabolite profile is compared to unveil markers which differ between animal cohorts (Fiehn, 2002; Dettmer et al. 2007). The acquisition of such a vast amount of data requires both bioinformatic tools to generate models which can distinguish the disrupted homeostatic state due to exogenous drug administration and predictive techniques that assign acquired data to an assumed response (Antignac et al. 2011; Courant et al. 2014). Recent research incorporating the whole profile of blood metabolites to discriminate cattle exposed to growth promoting agents has been described (Regal et al. 2011; Dervilly-Pinel et al. 2012; Graham et al. 2012; Nzoughet et al. 2015a), yet progress towards applicable screening approaches is as yet unrealised. Metabolites contributing to differentiating profiles have been investigated (Riedmaier et al. 2009; Pinel et al. 2010; Dervilly-Pinel et al. 2012) but their reliability is often obscured by biological and environmental conditions and the specific relevance of metabolite profiles to individual growth promoter treatments is not clear.

The focus of the current study has centred on the detection of metabolomic markers significant to the misuse of glucocorticoid (dexamethasone and prednisolone) and oestradiol compounds in bovine animals for meat enhancement purposes. Glucocorticoid agents are readily available for therapeutic veterinary applications but may be misused through long-term low-dose regimes which sustain animal health whilst encouraging lean meat production (Antignac et al. 2001; Cannizzo et al. 2011). The use of oestradiol for growth promoting purposes is currently prohibited by the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) (Directive, 2003),

and whilst effective monitoring procedures have been established, it's availability outside the EU is thought to contribute to a black market of illicit use (Courtheyn et al 2002; Regal et al. 2012) with administrations difficult to distinguish from variable endogenous levels in cattle (Regal et al. 2011). We for the first time unveil the bovine plasma metabolome changes, detected by ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS), significant to the administration of oestradiol, dexamethasone or prednisolone. The results illustrate the use of advanced statistical models incorporating ions altered by various treatment types to predict growth promoter exposure, whilst putative identifications highlight the possible underlying metabolite functions specific to administered compounds.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

LC-MS grade methanol (MeOH) and formic acid (HCOOH) were purchased from Sigma Aldrich (UK). Leucine enkephalin (Leu-Enk) was sourced from Waters (UK) and ultra-pure water (18.2 MΩ cm⁻¹) was generated in-house using a Millipore system (Millipore, USA).

2.2. Experimental design and plasma sample collection

Samples were obtained from an experimental treatment study using growth promoting regimes reflective of suspected on-farm practices conveyed in the literature (Courtheyn et al. 2002; Cannizzo et al. 2008; De Maria et al. 2009). Authorized by the Italian Ministry of Health and bioethics committee of the University of Turin, the study cohort consisted of twenty four male *Charolais* cattle aged 17-22 months old randomly assigned to four treatment groups: Group O (*n*=6) received 0.01 mg/kg intramuscular injection of 17β-oestradiol-3-benzoate (Sigma-Aldrich, Milan, Italy) weekly on day 12, 19, 26, 33 and 40; Group D (*n*=6) were administered an oral dose of 0.7 mg/day dexamethasone-21-sodium phosphate (Desashock Fort Dodge Animal Health, Bologna, Italy) for 40 days; Group P (*n*=6) were given 15 mg/day prednisolone acetate orally (Novosterol, Ceva Vetem SpA, Milan, Italy) for 30 days; Group C (*n*=6) were control untreated animals. All animals were kept in separate housing and fed a diet of silage, corn and hay alongside a commercial protein supplement and water. Blood was collected via the jugular vein on days 0, 7, 25, 35, 43 and at slaughter (day 49) using EDTA tubes for plasma preparation (centrifugation at 2,000 x *g* for 20 min) which was stored at -80°C.

2.3. Plasma sample preparation for metabolomic profiling

Minimal sample preparation was carried out on twenty four plasma samples collected on day 25 of the animal study to ensure intact metabolite coverage with samples randomized during analysis to avoid bias in preparation. A volume of 100 μL of plasma was centrifuged at 1,000 $\times g$ for 5 mins and extraction conducted by addition of 400 μL ice cold MeOH ($<20^\circ\text{C}$), vortexing briefly and holding on ice for 20 mins. Samples were subsequently centrifuged at an increased speed of 15,000 $\times g$ for 10 mins to separate the proteinaceous pellet. The supernatant was removed and evaporated to dryness in a Savant™ SpeedVac™ concentrator (Thermo Scientific, USA) operating at 40°C , and reconstituted in 50 μL ultrapure water with 5 mins vortexing followed by filtering through a Costar® Spin-X 0.22 μm tube filter (Sigma Aldrich, UK) under centrifugation at 15,000 $\times g$, 4°C for 2 mins. 100 μL from each of the plasma samples under investigation were pooled and similarly prepared for quality (QC) purposes. Filtered samples were stored at -80°C in a LCGC Certified Clear Glass (12 x 32 mm) autosampler vial (Waters, UK) prior to UPLC-HRMS analysis.

2.4. UHPLC-QToF-MS metabolomic plasma profiling

Chromatographic separation was performed using an Acquity™ UPLC system (Waters, UK) comprising of a stainless steel Aquity UPLC® HSS T3 analytical column (100 x 2.1mm, particle size 1.8 μm) (Waters, UK). The column temperature was maintained at 45°C with an autosampler at 6°C . A volume of 7.5 μL sample was injected at a flow rate of 0.4 mL/min and analysed over a 20 min gradient consisting of mobile phase A, $\text{HCOOH}:\text{H}_2\text{O}$ (1:1000, v/v), replaced by mobile phase B, $\text{HCOOH}:\text{MeOH}$ (1:1000, v/v). The initial gradient was set at 1% B for 2 mins increasing to 70% B, by 7 mins to 99% B at 16.25 mins, maintained for 2.25 mins before returning to 1% B for the remaining 1.5 mins. Wash

solvent was prepared with H₂O:MeOH (1:1, v/v), the seal wash was made up of H₂O:MeOH (95:5, v/v) and the purge wash consisted of HCOOH:H₂O (1:1000, v/v).

2.5. System calibration and quality control checks

A reference standard (Waters, UK) consisting of 2ng/μL nine component mix of acetaminophen, caffeine, sulfaguanidine, sulfadimethoxine, Val-Tyr-Val, verapamil, terfenadine, Leu-Enk and reserpine, was injected prior to each run (*n*=3) to ensure retention time and mass accuracy. The UHPLC-QToF-MS system was equilibrated with sodium formate at a flow rate of 5 μL/min with column equilibration via injection of ten replicates of pooled plasma prior to analysis and intermittent injections of pooled QC plasma throughout the run. Leu-Enk was introduced at 1 ng/μL in H₂O:MeCN:HCOOH (50:50:0.1, v/v/v) to compensate for mass shift.

2.6. Mass spectrometry data acquisition and processing

The UHPLC system was coupled to a Xevo® G2 Q-ToF mass spectrometer and controlled via v4.1 MassLynx™ software (both from Waters, UK). Q-ToF-MS data was acquired in resolution mode. Acquisition was conducted using positive electrospray ionization mode (ESI+) with the capillary voltage set at 1 kV and the cone gas flow of 50 L/h. The source temperature was set at 120°C with 60 AU offset and the desolvation gas set at 450°C with flow rate of 850 L/h. Nitrogen was employed as the desolvation and cone gases. Data was acquired in continuum mode using MS^E with scan time 0.1 sec over 50-1200 Da under 4 eV low energy and a ramp of 20-35 eV at high energy. Lockmass calibration during data acquisition was set at capillary voltage 1.40 kV and collision energy 20 eV with dual point correction of Leu-Enk ([M+H]⁺=278.1141, 556.2771 Da) scanning for 0.2 sec at 30 s

intervals with mass window ± 0.5 Da. UHPLC-HRMS raw data files were exported for pre-processing into Progenesis QI® software (Waters, UK). Chromatograms were aligned to a reference QC run and selected adducts included $[M+H]^+$, $[M+H-H_2O]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+H-2H_2O]^+$, and $[M+CH_3OH+H]^+$ with 2.5 AU filter. Peak picking was set to exclude ions eluted before 0.5 mins (1%) and after 14 mins (92%) at 1% and 92% organic solution, respectively. Deconvolution was applied to detect ions which may be formed from the same compound. The raw data was normalized for all compounds and exported in .CSV format for further analysis reporting selected ions by their accurate mass and retention time pair (AMRTP). Ions which demonstrated relative standard deviation (RSD) greater than 30% in QC pools were discarded from analysis. An 80% rule was also applied to eliminate any ions which were not detected in more than 80% of the samples analysed; that is, where a zero value was obtained in more than 20% of the sample set analysed, it was considered spurious and excluded from the dataset.

2.7. Analysis of plasma metabolite ions discriminating between growth promoter treatment regimes

2.7.1. Univariate statistics

Acquired plasma metabolome datasets were first analysed using Excel 2010 (Microsoft Office, USA) for conventional statistical comparison of treated and untreated groups. Three technical replicates were averaged to provide a peak abundance of six animals per treatment group for the respective ions detected. Additionally a lower limit of 500 for the peak output was applied whereby ions showing average abundance <500 across the respective treatment groups were excluded as indistinguishable from background noise. The data was assessed for normal distribution by regression analysis and each treatment

group was tested for unequal variance via *F*-test against the control group. Significance of $p < 0.05$ was then determined using a two-tailed student's *t*-test comparing the control untreated group to each treated animal group, where type 2 was applied for homoscedastic variance ($F > 0.05$) and type 3 when heteroscedastic ($F < 0.05$). Those ions showing significant difference in abundance when compared to the control output were considered useful for further analysis.

2.7.2. Multivariate statistics

SIMCA version 13.0 (Umetrics, Sweden) was used for multivariate analysis of metabolomic profiles of plasma from various treatment groups to highlight discriminatory ions. The normalized value outputs from Progenesis QI® for each sample injection, inclusive of all metabolites (<30% RSD), were subject to pareto scaling and projections were made between control and respected treated animal sets. Principal Component Analysis (PCA) enabled unsupervised separation of the test samples against the QC injections to ensure quality control samples were clustered together and check that the technical replicate injections were similarly aligned. Test samples were assigned as either control or treated for subsequent supervised analysis via orthogonal partial least squares discriminant analysis (OPLS-DA). Having obtained good prediction (Q^2) and separation (R^2) via OPLS-DA, S-plot and variable importance of projection (VIP) plots were utilized to select those ions contributing most to the predictive components. Markers were selected based on having a VIP score greater than 1 and subsequent jack-knifed confidence intervals with coefficient (cvSE) less than the VIP score. These were reviewed via S-plot representing positive or negative correlation $p(\text{corr})$ against covariance $p[1]$ with those plotted on the outer edges of the graphical area ($p[\text{corr}] > 0.5$) considered to have strong discriminatory power. These ions were then employed to create a new OPLS-DA model

verifying their predictive ability with 100 rounds of permutation testing alongside cross-validation via CV-ANOVA.

2.8. Metabolite identification

Selected AMRTPs were confirmed as peaks extractable from the raw chromatogram using MassLynx™ and the theoretical elemental composition of the selected feature determined (Kind et al. 2007; Watson, 2013). Mass uncertainty was set at 5 mDa or 10 ppm for those ions with MW ≤ 400 Da or >400 Da, respectively, and elements were restricted to C, H, O, N, P and S. The mass of the parent compound was then searched against known databases (METLIN, HMDB, PubChem) to make putative identifications in consideration of the isotope pattern and subsequent charged state with adducts confirmed against the mass spectrometry adduct calculator provided by Fiehn Lab (<http://fiehnlab.ucdavis.edu/>). Biological contributions were determined from subsequent PubChem classification (NCBI, USA) and corresponding KEGG (Kanehisa Laboratories, Japan) and LIPID MAPS (www.lipidmaps.org) pathway analysis.

3. RESULTS AND DISCUSSION

Metabolite markers specifically altered within the plasma of cattle in response to glucocorticoid and oestradiol treatment regimes were successfully reported using UHPLC-HRMS metabolomics profiling.

3.1 Assessment of plasma metabolome profile output

A comparison of the profile of metabolites detected in bovine plasma following UHPLC-HRMS analysis is displayed in Figure 1 with base peak ion chromatogram (BPI) obtained from MassLynx™ representing control, oestradiol, dexamethasone and prednisolone plasma metabolomes. Whilst it is not possible to discern obvious differences in the resulting plasma metabolome profile from the BPI, it is anticipated that any minor deviations from the untreated state could reflect xenobiotic influence and respective chromatograms were subsequently analysed via Progenesis QI® software. This enabled automated alignment, peak picking and mining of the generated metabolome dataset. The plasma metabolomic profile of each sample injection ($n=72$) was displayed as an ion intensity map and replicate injections were combined and matched across treatment groups for quality assurance. Resulting chromatograms conferred 3522 features detected across all runs, which were further deconvoluted to 3088 ions. Peak abundance was normalized and elimination of those ions displaying a $RSD>30\%$ in QC injection lead to a reduction of the dataset to 1364 ions. Proceeding with the application of the 80% rule, additional falsities were omitted assembling a final list of 751 metabolites suitable for multivariate analysis.

3.2 Metabolite fingerprint modelling via multivariate classification

Unsupervised principal component analysis (PCA) was initially employed to compare the metabolome profiles of test samples to the QC samples from the 3088 detected features (Figure 2a). QC samples were tightly clustered demonstrating stability of the run and individual test replicate injections were similarly aligned showing repeatability of the platform performance. PCA was then used to ascertain if study groups could be classified from an unsupervised prospective based on the 751 ions of interest (Figure 2b). However, separation of control and treated profiles was not clearly distinguishable and subsequently supervised separation was applied. An OPLS-DA model was constructed from 751 variables and 63 observations based on 3 components with the control untreated profile plotted against each treated metabolome profile (Figure 3a) and demonstrated good fit based on % variation of the data set ($R^2X = 0.907$, $R^2Y = 0.978$) with potential to predict new data ($Q^2 = 0.916$). Overfitting of the OPLS-DA dataset was avoided by allocating principal components based on 7 rounds of cross-validation. Furthermore, assessment of each treatment group to the control via OPLS-DA based on 751 variables (Figure 3b-d), showed good separation and predictive ability of oestradiol ($R^2Y = 0.993$, $Q^2 = 0.940$), dexamethasone ($R^2Y = 0.986$, $Q^2 = 0.943$), or prednisolone ($R^2Y = 0.890$, $Q^2 = 0.723$) plasma profiles. Interestingly, both glucocorticoid treatments demonstrated similar patterns of separation from the untreated group yet distinctive metabolome changes are further discussed.

3.3 Analysis of selective ions contributing to separation

The variables contributing most to the separation of untreated and treated profiles were assessed via the variable importance of projection (VIP) plot which showed 135 ions with $VIP > 1$ within the confidence intervals⁴. Analysis of the VIP scores and subsequent S-plots

for each treatment type as shown in Figure 4, revealed 56 ions relevant to oestradiol treatment, 45 ions discriminating dexamethasone treatment and 58 significant to prednisolone treatment profiles. Shared and unique structure (SUS) plots were used to illustrate ions comparable across the treated profiles (Figure 5) culminating in 99 features of interest.

3.4 Assessment of the predictive ability of the multivariate model

The selected ions ($n=99$) were used to generate a new OPLS-DA model (Figure 6a) capable of discriminating treated from untreated metabolome profiles. It is anticipated that a model could indicate the use of either oestradiol, dexamethasone or prednisolone. The predictive ability of the model was tested by computing 100 rounds of iteration testing. This automatically assigned permuted points to the left with new observations lower than the original untreated on the right (see figure 6b). The regression showed sufficient separation of newly generated observations demonstrating good prediction with Q2 below the X-axis (-0.62). The model was further tested by cross validation based on 2/3 of the dataset used to correctly assign the remaining 1/3. Assessment of the significance of the model by CV-ANOVA based on F -distribution of the prediction error with one degree of freedom showed significant regression ($p<0.01$) with p -value of 1.73×10^{-18} . Similar assessment of the separate treatment group models for oestradiol, dexamethasone or prednisolone prediction showed significant classification ($p<0.01$) from CV-ANOVA.

3.5 Statistical analysis of discriminatory ions

Further assessment of the metabolic changes found 32 features at an increased level ($FC>1.5$) and 29 down-regulated ($FC<0.5$) by more than 50% in response to the various

compound administrations (Table 1a and 1b), respectively. Notably ten ions were commonly increased in all treatment groups, whilst seven ions were found to be depressed. Finally, comparison of the metabolome profile of each treatment group to the control group via student's *t*-test analysis revealed 24 ions which were significantly ($p < 0.05$) altered across the biological replicates (Table 2) and were identified as possible biomarkers of the respective growth promoter treatments.

3.6 Assignment of putative identification of ions of interest

The use of both high- and low-collision energies during the mass spectrometry data acquisition provided fragment rich spectra for putative identifications of the features of interest. The spectrum of each ion was extracted from a representative chromatographic peak highlighting the precursor ion. Examination of the isotope ratio confirmed the charge state as single (+), double (++) or triple (+++) in deduction of the visible adduct formation. The putative mass was matched against the fragmented spectrum and resulting identities of the 24 metabolites of interest (Table 2). The maximum mass difference observed was 12 mDa with exception of AMRTP 4.41_344.8505 m/z , 5.80_741.3737 m/z and 6.07_618.6912 m/z whose mass differences were 36.1, 16.6 and 16.4, respectively. However, these adducts represent larger compounds where additional fragments available from function 2 data were incorporated to check tentative assignments.

3.7 Biological classification of identified marker metabolites

Putative identifications reveal the majority of functional roles relating to lipid metabolism via glycerophospholipid and sphingolipid pathways (see Fig 7) as well as fatty acid

metabolism and additional roles such as acylcarnitines, dipeptides, eicosanoids and vitamin or steroid derivatives. These findings complement biomarker candidates previously highlighted by proteomic investigation (Kinkead et al. 2015) implicating anabolic interactions with apolipoproteins and vitamin-D-binding proteins as responsible for the underlying biological mechanisms. An example of the expression levels of specific markers displayed by the differing treatment groups is shown in Figure 8 and are further discussed below.

3.7.1 Phospholipids

Resulting plasma metabolite identifications indicate cytidine diphosphate diacylglycerol (CDP-DG) and other glycerophospholipids including those bound to glycerol (PG, DG), inositol (PI), choline (PC) and ethanolamine (PE) as significantly perturbed by growth promoter treatment regimes. They are members of the G-protein coupled receptor family previously implicated as responsive to steroid influence (Prossnitz et al. 2008)) and significantly altered by growth promoter treatment (Nzoughet et al. 2015b).. These glycerophospholipids are known to play an important role in the generation of triacylglycerides from glycerol-3-phosphate by acting on phosphatidic acid and contributing metabolic functions (Stapleton et al. 2011). However plasma levels of PG (18:4(6Z,9Z, 12Z,15Z)/12:0) were also shown to be reduced by growth promoter treatment in this study as it is known to be cleaved to form diacylglycerol. The fluctuating levels are probably due to the relative transformation state but may also be dependent on the agent administered. Others (Kitson et al. 2013) found increased plasma phospholipid levels in rats supplemented with 17 β -oestradiol whilst Nzoughet et al. (2015b) reported a decrease in PG, PI, PA, PE, PC upon administration of trenbolone acetate. It is known that these phospholipids are important in the generation of

triacylglycerides and subsequent metabolic functions (Stapleton et al. 2011). Additionally LysoPC was notably increased in the study and is formed in plasma by a specific enzyme system, lecithin-cholesterol acyltransferase (LCAT) which catalyzes the transfer of fatty acids to free cholesterol bound to high/low density lipoproteins (H/LDL). This complements previous proteomic analysis showing increased levels of apolipoproteins A1 and AIV (Kinkead et al. 2015).

3.7.2 Sphingolipids

Treatment of cattle with oestradiol, dexamethasone or prednisolone resulted in a dramatic increase in the level of plasma sphingolipids in this study. Sphingolipids have been shown to modulate the steroid hormone biosynthetic pathway at multiple levels, including gene expression, steroidogenic activity and act as second messengers in signalling cascades (Lucki, Sewer 2008; Sabourdy et al. 2008). Studies have shown demonstrable increased levels associated with insulin resistance and contributing metabolic disorders where glucocorticoids are known to have large and specific effects on sphingolipid expression (Holland, Summers, 2008). Specifically, ganglioside GT1b, 6Gal β 1, Gal α 1-3, GM3 were increased most by prednisolone administration in this study with observable fold change differences ranging from 3-11 times the peak output from the untreated plasma metabolite profile. These gangliosides make up the cell membrane and are involved in signal transduction mediated by the liver contributing to lipoprotein sialylation and cholesterol efflux through inhibition of glycerophospholipids (Millar, 2001, Subbaiah et al. 1993). Previous reports indicate dysregulation of sphingolipids having profound effects on glycerophospholipid expression in plasma (Rodrigues-Cuenca et al. 2017). Sphingolipids can be found in all tissues and biofluids since they contribute largely

to the immune response (Garcia-Barros et al. 2015). Furthermore, gangliosides are known to inhibit pro-inflammatory signals (Miklavcic et al. 2012) which may explain why they are increased herein more so by prednisolone due to its anti-inflammatory action.)

3.7.3 Fatty acid synthesis

An increase in 6-oxocyclohex-1-ene-1-carboxyl-CoA was displayed across all treatments in this study and has not previously been reported relevant to the bovine growth promoter response. We know acyl-Coenzyme A plays a key role in fatty acid metabolism as well as lipid synthesis and expression levels are altered by anabolic influence for energy production (Zhao et al. 2016; Pietrocola et al. 2015). Whilst oestradiol administration resulted in the greatest increase in CoA in this study, the levels expressed were shown to be most significantly evident in dexamethasone treated animals with lower levels displayed by the prednisolone treatment group. Other studies have found increased expression of the CoA gene after administration with prohormone dehydroepiandrosterone for the purpose of growth promotion in cattle (Rijk et al. 2010). Acyl CoA is also influenced by glucose homeostasis with increased expression resulting in high uptake of fatty acids in adipose tissue and increased triacylglycerol synthesis (Zhao et al. 2016). Fatty acid metabolites *N*-stearoyl tyrosine and hypusine were notably decreased after growth promoter treatment herein and may reflect the feedback redirection of acyl thioesters from plasma via CoA binding proteins (Faergeman, Knudsen 1997).

3.7.4 Acylcarnitines

The level of acylcarnitines in the plasma metabolome of growth promoter treated animals was found to be reduced in comparison to that of untreated animals. Specifically, heptanoylcarnitine was reduced by up to 73% and found to be significantly depressed by oestradiol treatment, whilst other stearidonyl carnitine and 12-hydroxy-12-octadecanoylcarnitine were found to be significantly reduced within the dexamethasone metabolome profile. Acylcarnitines are predominantly derived from β -oxidation of fatty acids and increased levels are known to contribute to fatty acid metabolism to meet energy demands whilst decreased utilization of fatty acids would lead to decreased acylcarnitine expression (Hoppel, 2003; Thompson et al. 2012). The regulation of acylcarnitines is interestingly mediated by CoA with accumulated response to inflammation, insulin sensitivity and other signal transduction events (McCoin et al. 2015).

3.7.5 Eicosanoids

An increase in the level of an eicosanoid was observed across all growth promoter treated plasma profiles. Eicosanoids are synthesised from arachidonic acid and are involved in autocrine and paracrine response (Nebert, Russel, 2002) operated via G protein receptors through lipid signalling pathways (Funk, 2001). They have not previously been implicated in the bovine growth promoter response but are known to be anti-inflammatory mediators (Salmon, Higgs 1987) and specifically leukotriene E3 was significant to prednisolone administration.

3.7.6 Additional metabolites

Interestingly, the dipeptide tryptophyl-proline was shown to be increased in the plasma of cattle exposed to growth promoter treatments in this study alongside isoleucyl-

tryptophan which was significant to the dexamethasone treatment group. Regal et al. (2014) also reported an increase in pyroglutamyl-phenylalaninedipeptide as a biomarker of oestradiol administration. Such increases may be attributed to roles in protein metabolism but their specific action in the growth promoter response remains unclear. Other metabolites of interest were found to be significantly reduced in response to dexamethasone treatment including a bile acid (Chenodeoxycholic acid glycine conjugate), vitamin D3 derivative (3'-O-Aminopropyl-25-hydroxyvitamin D3) and steroid metabolite (1'H-5alpha-Cholest-2-eno[3,2-b]indole), which is indicative of additional biological pathways affected by corticosteroid metabolism. Moreover, an increase in 3-methylene-indolenine within oestradiol and prednisolone treatment metabolome profiles is not clear since it functions as a catalyst in the formation of reactive intermediates from pneumotoxic chemicals via cytochrome P450. An additional marker ion (6.80_989.5636m/z) shown to be significantly increased in response to oestradiol treatment could not be identified from available metabolite databases.

4. Conclusions

Given the many issues which compromise the effective and sensitive monitoring of illegal growth promoter use in food production, it is clear that new strategies are required to more confidently determine cases of anabolic exposure that do not solely focus on concentration levels of drugs or their metabolites which can be eliminated rapidly or are autogenously present. The work herein demonstrates the capability of metabolomic screening methods to identify bovine animals subjected to various types of growth promoting treatment regimes. This is the first investigation of alterations to the metabolome detectable within plasma relevant to prednisolone, dexamethasone or

oestradiol administrations, with 58, 45 and 56 ions observed to be significantly altered in response to growth promoter treatments. Predictive modelling was shown to successfully discriminate between the metabolome profiles of plasma from treated and untreated animals based on 99 perturbed metabolite ions. Putative identification of specific metabolites enables the development of candidate markers significant to oestradiol, dexamethasone or prednisolone administration. These were mostly attributed to sphingolipid and glycerophospholipid pathways relevant to fatty acid synthesis with implications on cholesterol transport, glycolysis and immune responses. The differing responses relative to the level of drug exposure is yet to be determined but it is anticipated that further development of these gangliosides, lipoproteins, Co-enzyme A, acylcarnitines and eicosanoids into rapid onsite screening tools could detect cases of drug misuse prior to confirmatory analysis.

5. ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Figure 1. REPRESENTATIVE BASE PEAK ION CHROMATOGRAM OF BOVINE PLASMA METABOLOME - from A) control, B) oestradiol, C) dexamethasone, D) prednisolone obtained from UHPLC-HRMS profiling in positive electrospray ionization mode across an increasing methanol gradient.

Figure 2. MULTIVARIATE DATA ANALYSIS OF METABOLITE PROFILES OF PLASMA FROM PCA SCORES PLOT - of A) unsupervised separation of QC vs test data based on 3088 detected features; B) unsupervised separation of control from treated plasma metabolite profiles based on 751 ions of interest.

Figure 3. OPLS-DA CLASSIFICATION OF FEATURES ($n=751$) ACQUIRED FROM PLASMA METABOLOME PROFILES - of A) untreated vs treated, B) untreated vs oestradiol, C) untreated vs dexamethasone, D) untreated vs prednisolone. Data was previously subjected to pareto scaling and separation shown is based on the first two components.

Figure 4. VIP PLOT OF CONTRIBUTING VARIABLES - highlighted from the OPLS-DA model. 135 ions demonstrating $VIP>1$ and $cvSE<VIP$ were selected for further analysis as potential marker metabolites.

Figure 5. CONSTRUCTED S-PLOT OF FEATURES ($n=751$) DETECTED FROM BOVINE PLASMA GENERATED BY OPLS-DA MODEL - comparing control untreated to A) oestradiol B) dexamethasone C) prednisolone profiles. Ions highlighted red possess $VIP>1$ and demonstrate strong discriminatory power with $p[1] >0.05$ and $p(\text{corr}[1])>0.1$. The

positively correlated features are upregulated whilst negative features correspond to down regulation in the treated state.

Figure 6. SUS PLOT OF THE TREATED PROFILES - separating control from oestradiol (M70), dexamethasone (M69), prednisolone (M71) highlighting ions of interest as previously selected from OPLS-DA models. Comparison of markers between A) oestradiol to dexamethasone, B) dexamethasone to prednisolone, and C) oestradiol to prednisolone. The variables lying across the diagonal are shared across the treatment types whereas the outer boxes reveal those markers specific to the treatment profile where red-outlined box denotes those specific to the y-axis model and the blue-outlined box denotes those relevant to the x axis model. The number of commonly altered ions are depicted in D) a venn diagram based on 99 ions of interest.

Figure 7. CLASSIFICATION OF SIGNIFICANT MARKERS - Elucidation of the biological contribution of the 24 metabolites identified show over a third are relevant to lipids of which function as either phospholipids or sphingolipids. Another third function as fatty acyl or acyl carnitines, with the remaining metabolites involved in cell signalling and metabolism, and only one metabolite remained unidentifiable.

771 TABLES

772 Table 1a **RESULTING IONS OF INTEREST** – found at **increased** levels in treated animals
773 compared to non-treated. Represented by AMRTP showing the average peak output
774 within the control untreated group and relative fold change >1.5 in red alongside the VIP
775 score and *p*-value determined from student's *t*-test (*n*=6) highlighted in green as
776 significant (<0.05). Ten ions are shown commonly upregulated across all treatment types.

777

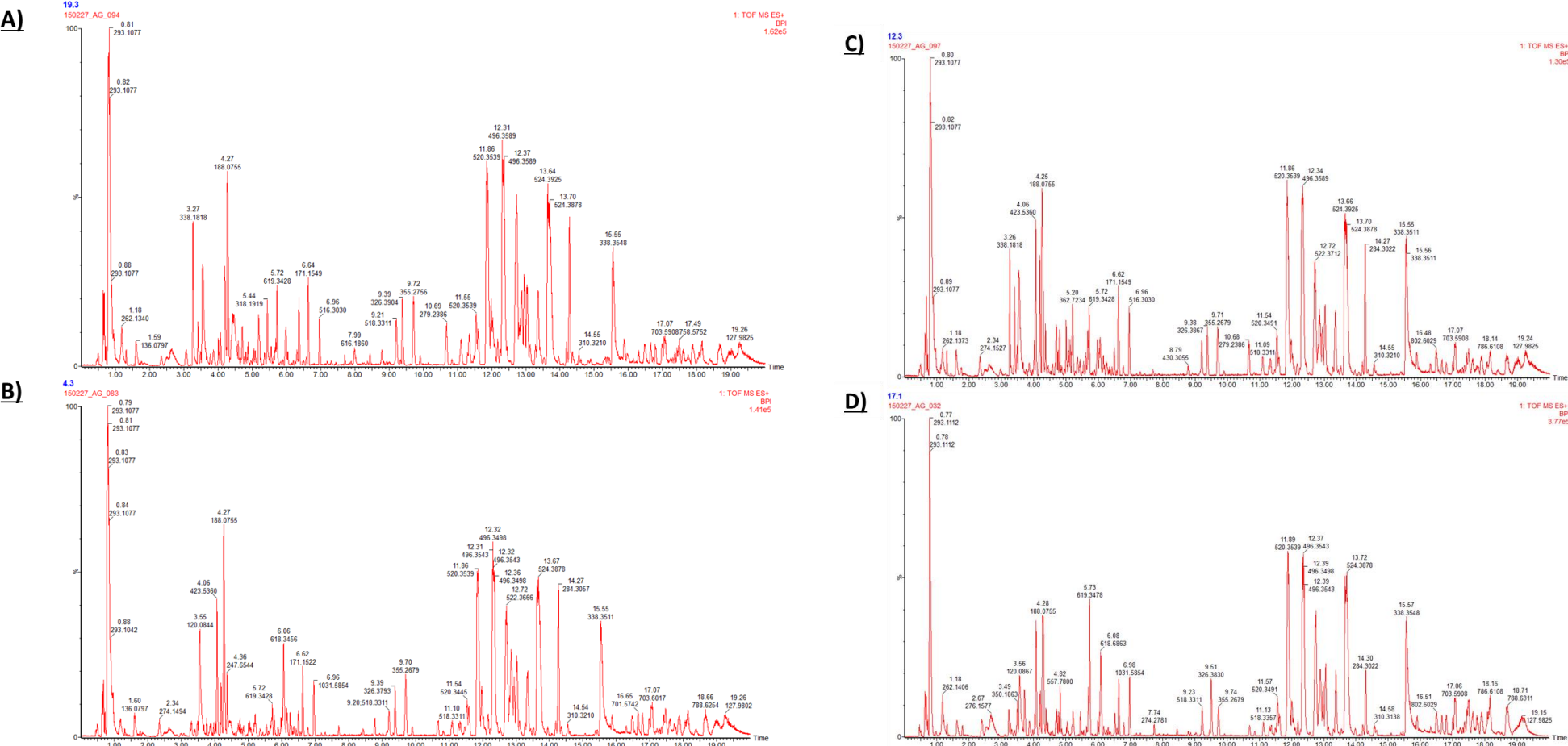
778 Table 1b **RESULTING IONS OF INTEREST** – found at **decreased** levels in treated animals
779 compared to non-treated. Represented by AMRTP showing the average peak output
780 within the control untreated group and relative fold change <0.5 in yellow alongside the
781 VIP score and *p*-value determined from student's *t*-test (*n*=6) highlighted in green as
782 significant (<0.05). Seven ions commonly decreased by all treatment types.

783

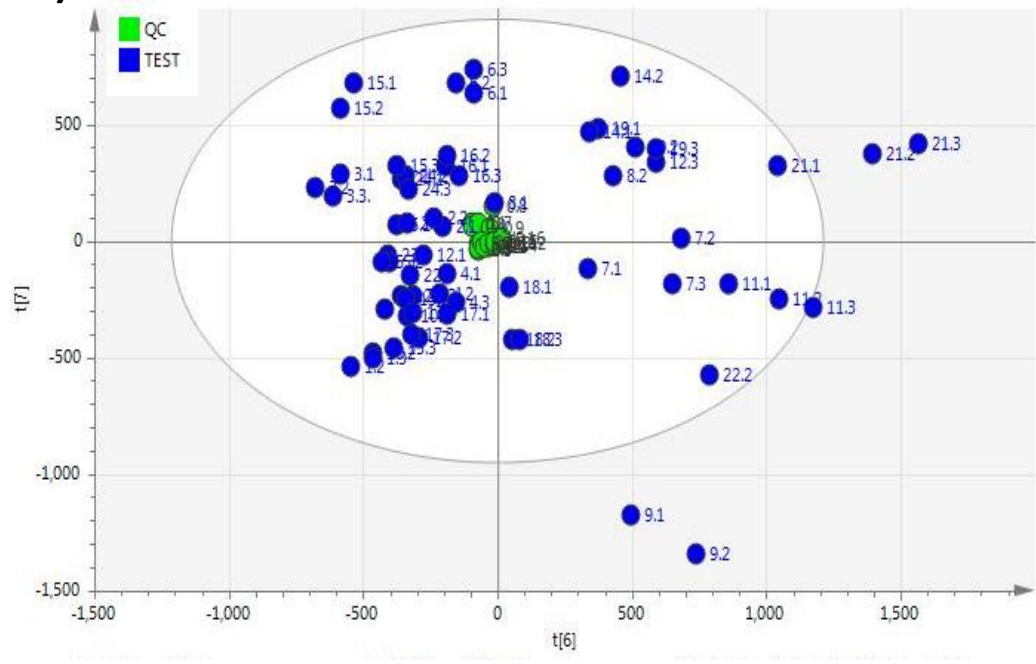
784 Table 2 **PUTATIVE IDENTIFICATION OF METABOLITES** – 24 plasma metabolites shown to
785 be cumulatively increased or decreased across the treatment profiles in comparison to
786 control untreated (*p*<0.05), were assigned identifications by accurate mass based on the
787 isotope charge (+, ++, +++) matching the formed adduct. Elemental composition analysis
788 predicted chemical formulae and corresponding assignments show a mass difference <11
789 mDa with exception of * ions which were confirmed by additional fragments present.

790 **FIGURE 1**

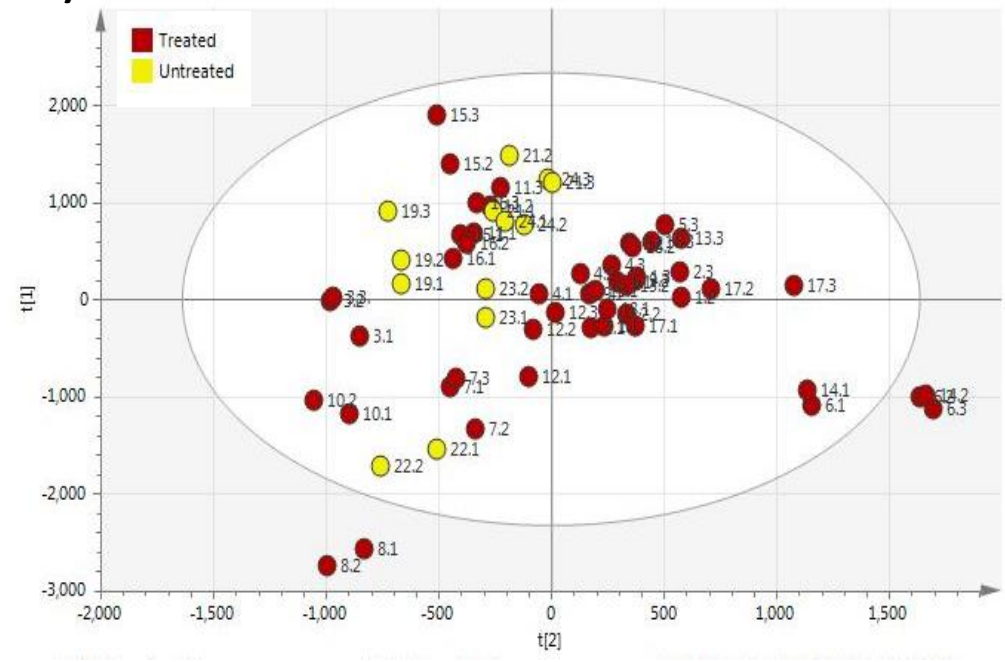
791



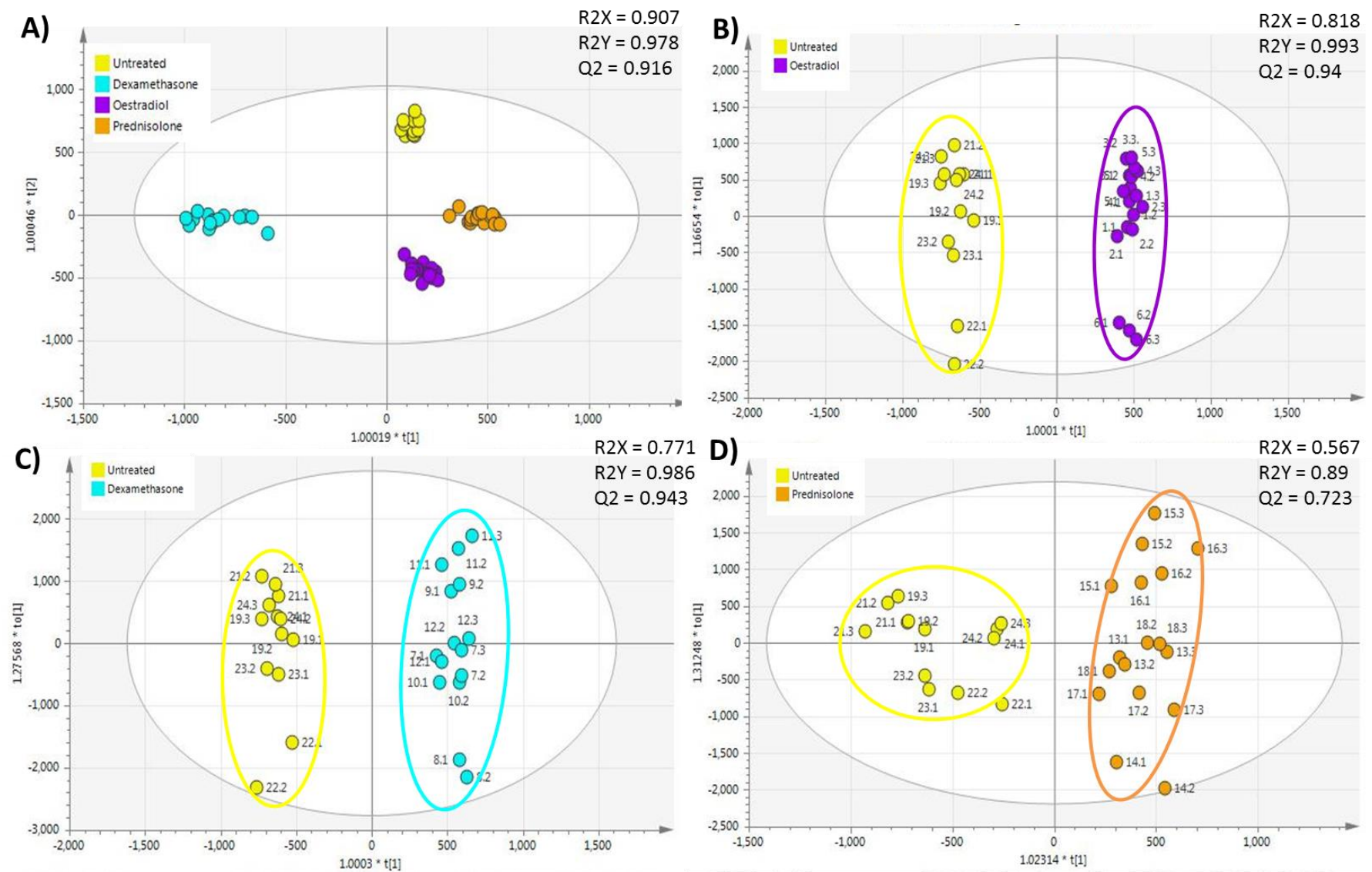
A)

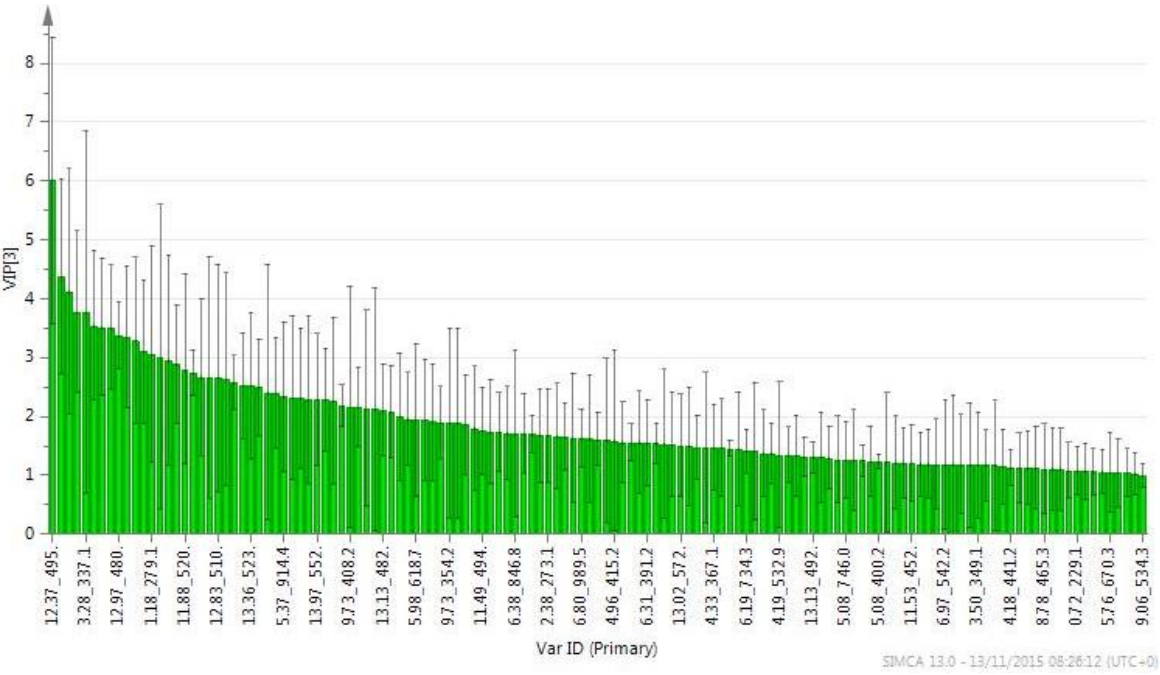


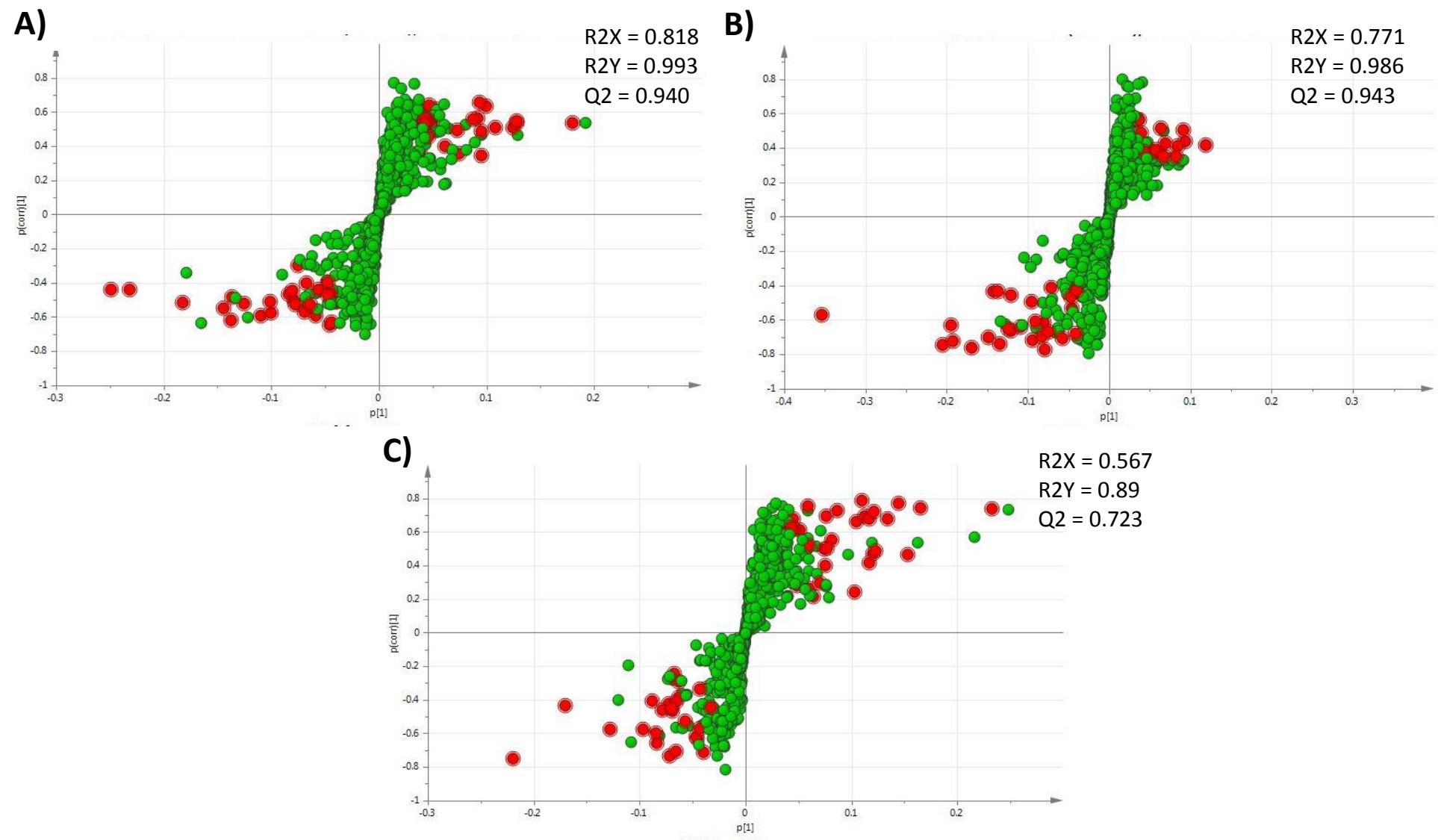
B)

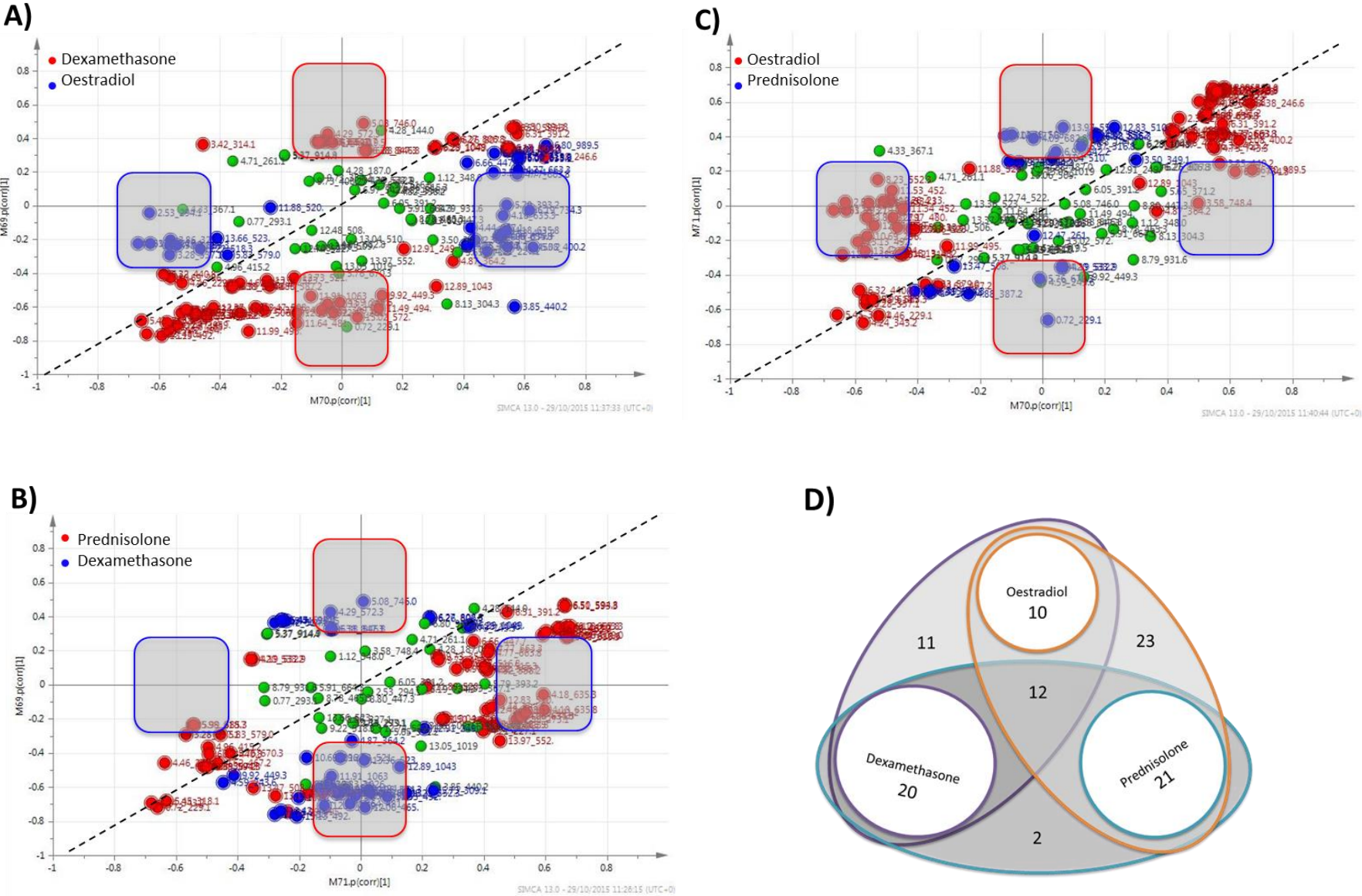


793 **FIGURE 3**

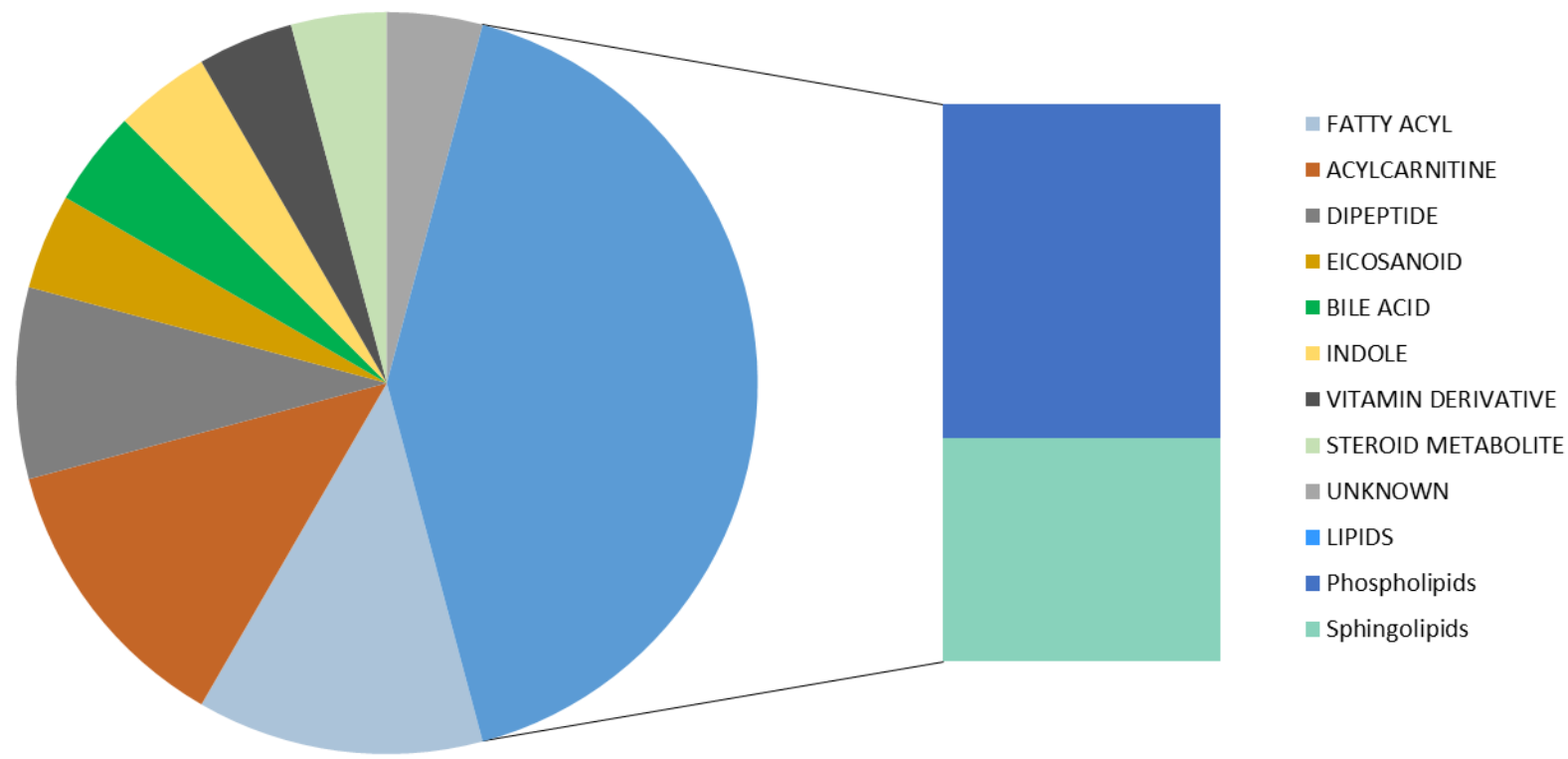








797 **FIGURE 7**



798 **TABLE 1a**

799

| AMRTP | UNTREATED Peak Output | Oestradiol | | Dexamethasone | | Prednisolone | | VIP |
|-------------------|-----------------------------|------------|-------|---------------|-------|--------------|-------|-----|
| | | FC | p | FC | p | FC | p | |
| 3.77_312.1489m/z | 660 | 4.07 | 0.066 | 3.88 | 0.038 | 1.81 | 0.250 | 1.2 |
| 4.41_344.8505m/z | 872 | 4.23 | 0.038 | 3.04 | 0.240 | 3.77 | 0.032 | 1.4 |
| 5.56_437.7554m/z | 1225 | 3.11 | 0.064 | 2.30 | 0.211 | 1.62 | 0.066 | 1.3 |
| 5.80_741.3737m/z | 246 | 10.75 | 0.065 | 6.76 | 0.293 | 8.72 | 0.028 | 1.3 |
| 6.08_463.0078n | 3415 | 6.87 | 0.092 | 1.97 | 0.419 | 5.04 | 0.022 | 1.2 |
| 6.07_618.6912m/z | 16072 | 3.40 | 0.074 | 1.52 | 0.484 | 3.34 | 0.013 | 2.4 |
| 6.17_662.8879m/z | 8069 | 2.43 | 0.117 | 1.55 | 0.357 | 2.56 | 0.017 | 2.0 |
| 6.18_442.2595m/z | 2180 | 2.32 | 0.137 | 1.60 | 0.345 | 2.89 | 0.031 | 1.5 |
| 6.31_391.2184n | 9545 | 1.91 | 0.097 | 1.72 | 0.251 | 1.51 | 0.207 | 1.6 |
| 6.51_593.3530n | 10254 | 2.90 | 0.067 | 1.95 | 0.192 | 1.97 | 0.024 | 2.3 |
| 5.04_338.0986m/z | 4161 | 1.53 | 0.063 | 1.53 | 0.147 | 1.30 | 0.181 | 1.1 |
| 6.27_806.8828m/z | 8805 | 1.93 | 0.370 | 3.62 | 0.207 | 1.33 | 0.664 | 2.1 |
| 6.80_989.5636m/z | 7695 | 2.04 | 0.028 | 1.51 | 0.372 | 1.26 | 0.648 | 1.6 |
| 4.38_246.6527n | 9928 | 1.98 | 0.037 | 1.34 | 0.443 | 1.83 | 0.067 | 1.7 |
| 6.01_560.3267m/z | 2057 | 1.96 | 0.123 | 1.15 | 0.774 | 2.02 | 0.032 | 1.1 |
| 6.59_130.0697m/z | 780 | 1.60 | 0.316 | 1.08 | 0.816 | 2.78 | 0.045 | 1.0 |
| 3.40_381.7018n | 3260 | 1.62 | 0.159 | 1.43 | 0.389 | 1.19 | 0.594 | 1.2 |
| 3.50_349.1792n | 9427 | 1.55 | 0.377 | 0.68 | 0.376 | 1.39 | 0.471 | 1.2 |
| 3.98_506.6963n | 3283 | 1.78 | 0.119 | 1.33 | 0.539 | 1.21 | 0.632 | 1.3 |
| 3.42_314.1654n | 10285 | 0.58 | 0.199 | 1.72 | 0.256 | 0.71 | 0.381 | 1.7 |
| 4.19_532.9512m/z | 8787 | 1.08 | 0.917 | 1.83 | 0.619 | 0.50 | 0.365 | 1.3 |
| 4.29_572.3757m/z | 15060 | 0.89 | 0.839 | 1.95 | 0.182 | 0.81 | 0.623 | 1.9 |
| 4.30_473.3004m/z | 1928 | 0.87 | 0.864 | 2.21 | 0.218 | 0.54 | 0.362 | 1.0 |
| 5.26_340.1144m/z | 1693 | 1.36 | 0.240 | 2.06 | 0.044 | 1.49 | 0.104 | 1.1 |
| 5.45_694.0660m/z | 5424 | 0.80 | 0.808 | 2.65 | 0.224 | 0.54 | 0.376 | 1.8 |
| 6.29_1049.5044n | 6745 | 1.10 | 0.805 | 1.64 | 0.411 | 1.47 | 0.442 | 1.5 |
| 6.38_846.8671m/z | 10334 | 0.88 | 0.847 | 2.12 | 0.371 | 0.67 | 0.622 | 1.7 |
| 4.07_423.5427m/z | 42359 | 1.37 | 0.259 | 0.75 | 0.367 | 1.67 | 0.130 | 4.4 |
| 4.08_477.5618m/z | 2226 | 0.82 | 0.636 | 0.62 | 0.376 | 1.83 | 0.143 | 2.3 |
| 4.68_285.6390n | 5848 | 1.34 | 0.363 | 1.12 | 0.708 | 1.52 | 0.165 | 1.2 |
| 4.82_556.7827n | 24546 | 1.19 | 0.684 | 0.98 | 0.960 | 1.51 | 0.236 | 3.2 |
| 13.97_552.4253m/z | 13655 | 1.08 | 0.747 | 0.73 | 0.373 | 1.62 | 0.196 | 2.3 |

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| AMRTP | UNTREATED Peak Output | Oestradiol | | Dexamethasone | | Prednisolone | | VIP |
|-------------------|-----------------------------|------------|-------|---------------|-------|--------------|-------|-----|
| | | FC | p | FC | p | FC | p | |
| 4.00_393.7204n | 11219 | 0.20 | 0.122 | 0.38 | 0.207 | 0.25 | 0.141 | 1.6 |
| 4.46_229.1634m/z | 31223 | 0.32 | 0.176 | 0.37 | 0.208 | 0.18 | 0.119 | 3.5 |
| 4.96_415.2549n | 8338 | 0.13 | 0.193 | 0.36 | 0.314 | 0.09 | 0.176 | 1.6 |
| 5.83_579.0046n | 4361 | 0.08 | 0.305 | 0.43 | 0.502 | 0.06 | 0.295 | 1.2 |
| 6.00_394.2106m/z | 2559 | 0.11 | 0.061 | 0.46 | 0.227 | 0.16 | 0.071 | 1.2 |
| 6.32_440.8133m/z | 3093 | 0.14 | 0.251 | 0.25 | 0.308 | 0.13 | 0.246 | 1.1 |
| 6.38_594.8657m/z | 8444 | 0.50 | 0.476 | 0.22 | 0.243 | 0.29 | 0.280 | 1.7 |
| 8.23_552.3425m/z | 7757 | 0.40 | 0.114 | 0.23 | 0.022 | 1.22 | 0.779 | 1.7 |
| 3.08_203.1468m/z | 3579 | 0.37 | 0.207 | 0.57 | 0.462 | 0.28 | 0.161 | 1.2 |
| 3.28_337.1815n | 70619 | 0.27 | 0.125 | 0.53 | 0.244 | 0.27 | 0.127 | 3.8 |
| 5.98_618.7759m/z | 20357 | 0.24 | 0.110 | 0.64 | 0.390 | 0.30 | 0.132 | 1.9 |
| 9.94_561.3523n | 1830 | 0.21 | 0.114 | 0.72 | 0.483 | 0.39 | 0.168 | 1.1 |
| 11.42_228.2382m/z | 2856 | 0.27 | 0.028 | 0.57 | 0.290 | 0.44 | 0.076 | 1.2 |
| 0.72_229.1280m/z | 1342 | 1.07 | 0.909 | 0.22 | 0.069 | 0.24 | 0.073 | 1.1 |
| 4.24_344.2161m/z | 4795 | 0.67 | 0.224 | 0.35 | 0.022 | 0.40 | 0.032 | 1.5 |
| 4.89_388.7325m/z | 1794 | 0.58 | 0.537 | 0.28 | 0.218 | 0.34 | 0.293 | 1.0 |
| 10.69_296.2437n | 21128 | 0.47 | 0.102 | 0.65 | 0.288 | 0.78 | 0.567 | 1.9 |
| 5.45_318.1921m/z | 10303 | 0.52 | 0.086 | 0.33 | 0.021 | 0.55 | 0.128 | 1.7 |
| 11.34_452.3281m/z | 27232 | 0.79 | 0.411 | 0.42 | 0.036 | 1.03 | 0.937 | 1.9 |
| 11.49_494.3394m/z | 14328 | 1.17 | 0.576 | 0.46 | 0.061 | 0.99 | 0.964 | 1.8 |
| 11.64_481.3341n | 37684 | 0.95 | 0.833 | 0.40 | 0.022 | 0.99 | 0.984 | 2.5 |
| 11.99_495.3517n | 74675 | 0.88 | 0.495 | 0.45 | 0.010 | 0.89 | 0.547 | 3.5 |
| 12.06_465.3376n | 60724 | 0.74 | 0.256 | 0.41 | 0.019 | 1.08 | 0.820 | 3.4 |
| 12.82_480.3605m/z | 37793 | 0.55 | 0.078 | 0.32 | 0.012 | 0.79 | 0.532 | 2.7 |
| 12.97_959.7157m/z | 9751 | 0.51 | 0.164 | 0.15 | 0.062 | 0.85 | 0.794 | 1.5 |
| 13.13_482.3789m/z | 26818 | 0.72 | 0.331 | 0.40 | 0.050 | 0.77 | 0.478 | 2.1 |
| 13.30_506.3807m/z | 22355 | 0.82 | 0.500 | 0.43 | 0.052 | 0.91 | 0.813 | 1.6 |
| 13.42_494.3792m/z | 24518 | 0.61 | 0.194 | 0.33 | 0.049 | 0.80 | 0.555 | 2.2 |
| 13.47_508.3950m/z | 16785 | 0.87 | 0.647 | 0.45 | 0.092 | 0.71 | 0.333 | 1.9 |

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| Treatment | FC | <i>p</i> | <i>m/z</i> | RT (min) | Isotope Charge | Elemental Composition | Mass | Adduct | MW | Mass Diff (mDa) | Compound | CID (PubChem) | Class |
|-------------------------------|----|----------------|------------|----------|-------------------------------------------|---------------------------------------------------------------------------------|-----------|-------------------------|---------|-----------------|--------------------------------------------|---------------|-----------------------|
| Oestradiol Prednisolone | + | 0.038 0.032 | 344.8505 | 4.41 | 344.8449+++ >516.7748++ >1032.5249+ | C ₄₈ H ₈₉ N ₃ O ₁₅ P ₂ | 1032.5661 | M+Na | 1009.58 | -36.10* | CDP-DG(18:0/18:0) | 53477922 | Glycerophospholipid |
| Dexamethasone Prednisolone | - | 0.022 0.032 | 344.2161 | 4.24 | 344.1996++ >687.3996+ | C ₃₆ H ₆₃ O ₁₀ P | 344.2152 | M+2H | 686.42 | 0.94 | PG(18:4(6Z,9Z, 12Z,15Z)/12:0) | 52926690 | Glycerophospholipid |
| Oestradiol | + | 0.028 | 989.5636 | 6.80 | 989.5685+ | C ₄₅ H ₉₀ N ₃ O ₁₄ P ₃ | | | | | Unknown | | |
| Oestradiol | + | 0.037 | 247.6600 | 4.38 | 247.6608++ >494.3061+ | C ₂₄ H ₄₈ NO ₇ P | 247.6657 | M+2H | 493.32 | -5.71 | LysoPC(16:1(9Z)) | 24779461 | Glycerophospholipid |
| Oestradiol | - | 0.028 | 228.2382 | 11.42 | 228.2345+ | C ₁₄ H ₂₇ NO ₄ | 228.2228 | M+2Na+H | 273.19 | 11.50 | Heptanoylcarnitine | 6426896 | Acylcarnitine |
| Dexamethasone | + | 0.038 | 312.1489 | 3.77 | 312.1359+++ | C ₂₈ H ₄₂ N ₇ O ₁₈ P ₃ S | 312.1502 | M+H+2Na | 889.15 | -1.29 | 6-Oxocyclohex-1-ene- 1-carboxyl-CoA | 442450 | Fatty Acyl Thioesters |
| Dexamethasone | + | 0.044 | 340.1144 | 5.26 | 340.1026+ | C ₁₆ H ₁₉ N ₃ O ₃ | 340.1058 | M+H | 301.34 | 8.55 | Tryptophyl-Proline | 23980# | Dipeptide |
| Dexamethasone | + | 0.022 | 552.3425 | 8.23 | 552.3353+ | C ₂₆ H ₅₄ NO ₆ P | 552.3400 | M+2Na-H | 507.37 | 2.50 | PC(O-18:1(11Z)/0:0) | 21672239 | Glycerophospholipid |
| Dexamethasone | + | 0.021 | 318.1921 | 5.45 | 318.1846+ | C ₁₇ H ₂₃ N ₃ O ₃ | 318.1812 | M+H | 317.17 | 10.93 | Isoleucyl-Tryptophan | 85823# | Dipeptide |
| Dexamethasone | - | 0.036 | 452.3281 | 11.34 | 452.3184+ | C ₂₅ H ₄₁ NO ₄ | 452.3370 | M+CH ₃ OH+H | 419.30 | -8.94 | Stearidonyl carnitine | 53477835 | Acylcarnitine |
| Dexamethasone | - | 0.022 | 482.3414 | 11.64 | 482.3299+ | C ₂₃ H ₄₈ NO ₇ P | 482.3476 | M+CH ₃ OH+H | 449.31 | -6.19 | Chenodeoxycholic acid glycine conjugate | 12544 | Bile acid |
| Dexamethasone | - | 0.010 | 496.3590 | 11.99 | 496.3498+ | C ₃₀ H ₅₁ NO ₂ | 496.3551 | M+K | 457.39 | 3.90 | 3'-O-Aminopropyl-25- hydroxyvitamin D3 | 12020395 | Secosteroid |
| Dexamethasone | - | 0.019 | 466.3449 | 12.06 | 466.3354+ | C ₂₅ H ₄₉ NO ₅ | 466.3503 | M+Na | 443.36 | -5.37 | 12-Hydroxy-12- octadecanoylcarnitine | 53481632 | Acylcarnitine |
| Dexamethasone | - | 0.012 | 480.3605 | 12.82 | 480.3422+ | C ₂₇ H ₄₅ NO ₄ | 480.3683 | M+CH ₃ OH+H | 447.33 | -7.80 | N-stearoyl tyrosine | 6710116 | Fatty Acyl Amide |
| Dexamethasone | - | 0.050 | 482.3789 | 13.13 | 482.3703+ | C ₃₃ H ₄₉ N | 482.3757 | M+Na | 459.39 | 3.18 | 1'-H-5alpha-Cholest-2- eno[3,2-b]indole | 17396442 | Steroid Metabolite |
| Dexamethasone | - | 0.049 | 494.3792 | 13.42 | 494.3606+ | C ₁₀ H ₂₃ N ₃ O ₃ | 494.3710 | 2M+3H ₂ O+2H | 455.40 | 8.15 | Hypusine | 65396 | Hydroxy Fatty Acyl |

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| Treatment | FC | <i>p</i> | <i>m/z</i> | RT (min) | Isotope Charge | Elemental Composition | Mass | Adduct | MW | Mass Diff (mDa) | Compound | CID (PubChem) | Class |
|--------------|----|----------|------------|----------|-----------------|-----------------------------------------------------------------|----------|-----------------------|---------|-----------------|------------------------------------------------------------------------|---------------|---------------------|
| Prednisolone | + | 0.028 | 741.3737 | 5.80 | 741.3531+ ++ | C ₉₈ H ₁₇₀ N ₄ O ₄₇ | 741.3571 | M+3Na | 2155.10 | 16.60* | Ganglioside GT1b (d18:1/20:0) | 53481259 | Sphingolipid |
| Prednisolone | + | 0.022 | 927.5272 | 6.08 | 927.5300+ + | C ₈₇ H ₁₅₄ N ₄ O ₃₆ | 927.5154 | M+H+Na | 1831.03 | 11.83 | NeuAcα2-6Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glcβ-Cer(d18:1/24:1(15Z)) | 44262970 | Sphingolipid |
| Prednisolone | + | 0.013 | 618.6912 | 6.07 | 618.6761+ ++ | C ₈₆ H ₁₅₆ N ₂ O ₃₇ | 618.6748 | M+2Na+H | 1809.04 | 16.44* | Galα1-3(Fucα1-2)Galβ1-3GalNAcβ1-3Galα1-3Galβ1-4Glcβ-Cer(d18:1/24:0) | 44260248 | Sphingolipid |
| Prednisolone | + | 0.017 | 663.3882 | 6.17 | 662.8710+ + | C ₃₃ H ₆₃ O ₁₃ P | 663.3879 | M+H-2H ₂ O | 698.40 | 0.29 | PI(12:0/12:0) | 52928414 | Glycerophospholipid |
| Prednisolone | + | 0.031 | 442.2595 | 6.18 | 442.2406+ + | C ₂₃ H ₃₉ NO ₅ S | 442.2622 | M+H | 441.25 | -2.69 | Leukotriene E3 | 6440643 | Eicosanoid |
| Prednisolone | + | 0.024 | 594.3603 | 6.51 | 594.3479+ + | C ₅₈ H ₁₀₄ N ₂ O ₂₁ | 594.3548 | M+H+Na | 1164.71 | 5.45 | Ganglioside GM3 (d18:1/9Z-18:1) | 20057284 | Sphingolipid |
| Prednisolone | + | 0.032 | 560.3267 | 6.01 | 560.3235+ + | C ₂₆ H ₅₂ NO ₇ P | 560.3322 | M+Na | 537.34 | -5.51 | PE(9:0/12:0)[U] | 40678# | Glycerophospholipid |
| Prednisolone | + | 0.045 | 130.0697 | 6.59 | 130.0663+ + | C ₉ H ₇ N | 130.0651 | M+H | 129.06 | 4.56 | 3-Methylene-indolenine | 170404 | Indole |

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